AD_____

Award Number: W81XWH-05-1-0535

TITLE: DNA Methylation as an Epigenetic Factor in the Development and Progression of Polycythemia Vera

PRINCIPAL INVESTIGATOR: Jean-Pierre Issa, M.D.
Jaroslav Jelinek, M.D., Ph.D.

CONTRACTING ORGANIZATION: The University of Texas
M.D. Anderson Cancer Center
Houston, TX 77030

REPORT DATE: October 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-10-2006	Annual	15 Sep 2005 – 14 Sep 2006

4. TITLE AND SUBTITLE

DNA Methylation as an Epigenetic Factor in the Development and Progression of Polycythemia Vera

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0535

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Jean-Pierre Issa, M.D.
Jaroslav Jelinek, M.D., Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of Texas
M.D. Anderson Cancer Center
Houston, TX 77030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Original contains colored plates: ALL DTIC reproductions will be in black and white.

14. ABSTRACT

Polycythemia vera (PV) is a clonal myeloproliferative disorder (MPD) affecting erythroid, myelomonocytic, and megakaryocytic lineages. An activating somatic mutation of JAK2 tyrosine kinase is present in the majority of PV patients but also in 50% of patients with essential thrombocythemia (ET) and myelofibrosis (MF). Additonal factors are presumed to affect the phenotype and progression of the disease. We studied DNA methylation as a possible epigenetic factor in the development and progression of polycythemia vera and related MPDs. We cloned 19 unique CpG islands in promoter/exon-1 regions of 15 known genes, and 4 predicted genes and annotated mRNAs as potentially hypermethylated in PV. We confirmed increased methylation of progesterone receptor and cadherin precursor (CDH13) in a subset of PV, MF and AML patients. We showed that a functional block of progesterone receptor in normal erythroid cells increases their sensitivity to erythropoietin. Silencing of these genes by methylation may contribute to disease development by altering the response of hematopoietic cells to proliferative stimuli or their interactions with stroma.

15. SUBJECT TERMS
Polycythemia vera; myeloproliferative disorders; epigenetics; DNA methylation

16. SECURITY CLASSIFICATION OF:				18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	25	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Cover……1

SF 298……2

Introduction……4

Body……8

Key Research Accomplishments……16

Reportable Outcomes……16

Conclusions……18

References……19

Appendices……20

DNA methylation as an epigenetic factor in the development and progression of polycythemia vera

Jean-Pierre Issa, M.D.

Grant MP04315

Study Site:

Department of Leukemia, University of Texas M. D. Anderson Cancer Center

1515 Holcombe Blvd., Houston, TX 77030

INTRODUCTION

Polycythemia vera (PV) is the most common myeloproliferative disorder with a yearly incidence of 28 per 1 million people and a slightly higher prevalence in males.[1] PV is characterized by clonal expansion of erythroid, myelomonocytic, and megakaryocytic lineages, erythrocytosis being the most prominent clinical manifestation of PV.[2] The disease is associated with a significant morbidity and mortality, including thrombotic and/or hemorrhagic events, and a risk of an evolution into myelofibrosis and leukemia.[3-5] An acquired activating V617F (1849G>T) mutation of *JAK2* tyrosine kinase has been recently found in the majority of patients with polycythemia vera (PV), in about half of those with essential thrombocythemia (ET) and myelofibrosis (MF),[6-10] and in 10-20% patients with chronic myelomonocytic leukemia, Philadelphia-negative CML, atypical or unclassified myeloproliferative diseases (MPD) and megakaryocytic leukemia.[11-13] It is not known what other factors determine the disease phenotype of PV, MF, and other

MPD, and what factors other than JAK2 lead to disease progression. Very little is known about epigenetic changes in PV. Epigenetic lesions have been recognized to be important in cancer, in particular in older individuals. Methylation of cytosines in the CpG sites clustered in the gene promoter regions results in epigenetic gene silencing, and acts as one of possible mechanisms of tumor suppressor inactivation in cancer.[14] Diverse myeloproliferative phenotypes caused by a single point mutation of JAK2 tyrosine kinase, lack of other genetic specific lesions in PV, and its association with higher age lead us to propose the hypothesis that epigenetic silencing may play a role in the pathogenesis of PV.

STATEMENT OF WORK

Task 1. Discover genes whose promoter-associated CpG islands are methylated in patients with polycythemia vera (PV), months 1-18:

a. Identify in the M. D. Anderson database all patients with PV for whom archived bone marrow biopsies are available (month 1).
b. Collect paraffin-embedded bone marrow biopsies on all patients (projected 100 patients, 10 cuts/month, months 1-10)
c. Collect existing blood samples from PV patients at M. D. Anderson, and from the external collaborator at Baylor College of Medicine (projected 50-60 patients per year, months 1-36).
d. Extract DNA from paraffin cuts (start month 1 – ongoing until all samples collected, months 1-10) and from blood samples (months 1-36).

e. Perform genome-wide screening for promoter-associated CpG islands differentially methylated in 15 patients with polycythemia vera in the polycythemic phase, 15 patients who developed myelofibrosis and 15 patients who transformed to leukemia. We will use Methylated CpG Island Amplification coupled with Representative Difference Analysis (MCA-RDA) as a screening method (months 2-18).

Task 2. Determine the methylation and expression profile of candidate genes in the polycythemic phase of PV, patients who developed myelofibrosis and patients who transformed to leukemia. Months 2-36.

a. Bisulfite treatment and PCR-based methylation analysis for all the genes discovered by MCA-RDA and candidate genes involved in growth factor signaling (months 2-20)
b. Analyze samples for gene expression by real time quantitative RT-PCR (months 13-36)
c. Statistical analysis of the collected data (months 21-22)
d. Validation of the results on prospectively collected samples (months 23-36)

Task 3. Begin exploring the function of the most promising genes using in vitro cultures and/or transfection experiments. Months 13-30.

a. Determine whether specific inhibition of candidate PV-methylated genes in normal cells would mimic the PV phenotype of hypersensitivity of

erythroid progenitors to erythropoietin (months 13-30).

b. In case the candidate genes are methylated and silenced in leukemic cell lines, we will restore their expression using standard gene transfection technology. The transfected cell lines will be examined for growth characteristics and in vitro differentiation. The effect of this transfection on the function of putative affected pathways will also be examined (months 13-30)

Task 4. To assess the prognostic significance of aberrant methylation in PV we shall perform retrospective multivariate analyses of the association of CpG island methylation with survival and probability of transformation to myelofibrosis or leukemia (months 24-36).

PROGRESS ON TASK 1

We have obtained 236 samples from patients with PV and PV-related myeloproliferative disorders. We extracted DNA from all samples and performed quantitative determination of JAK2 1849G>T (V617F) mutation by pyrosequencing (Fig. 1).

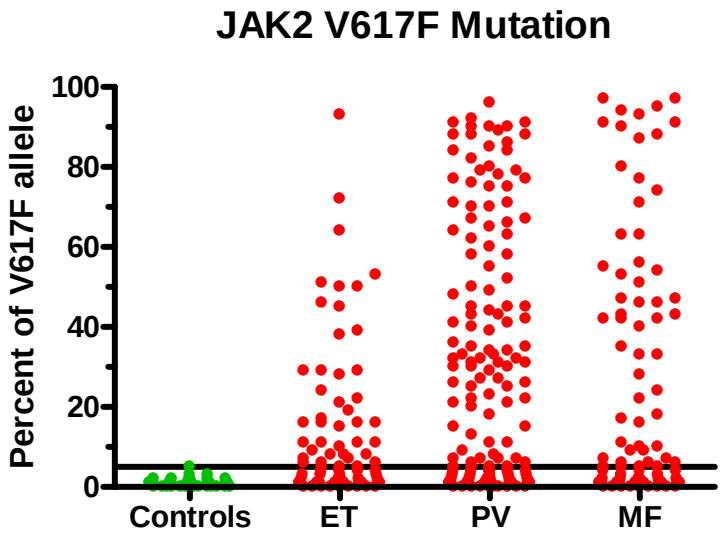

Figure 1. Quantitative determination of JAK2 V617F (1849G>T) mutation by pyrosequencing. None of 43 controls had values of mutant allele over 5% threshold (horizontal solid line). JAK2 mutation was detected in 39/74 (53%) ET patients, 98/138 (71%) PV patients and 50/88 (57%) MF patients.

We performed initial genome-wide screening by methylated CpG island amplification coupled with representative difference analysis MCA/RDA[15] for methylated promoter-associated CpG islands in granulocytes isolated from a PV patient. Cloning and sequencing of 200 clones revealed 19 unique CpG islands in promoter/exon-1 regions of

15 known genes, and 4 predicted genes and annotated mRNAs as potentially hypermethylated (Table 1).

Genes Cloned As Differentially Methylated in PV

Chromosome	Gene name	Description
1q23.3	CAPON	Adapter protein linking nNOS to specific targets
1q44	FLJ45717	Hypothetical protein
4p16.3	DGKQ	Diacylglycerol kinase
5q31.2	JMJD1B	Nuclear protein 5qNCA
5q35.2	SNCB	Beta-synuclein
6q15	BC037581	mRNA
10q11.21	GALNACT-2	Chondroitin N-acetylgalactosaminyltransferase
11q22.1	PGR	Progesterone receptor
12q23.3	CHST11	Chondroitin 4 sulfotransferase 11
12q24.12	LNK	Lymphocyte specific adapter protein Lnk
14q32	BC043593	mRNA
15q22.2	NLF1 exon2	Hypothetical protein
16p11.2	MGC2474	Hypothetical protein
16q23.3	CDH13	Cadherin 13 preproprotein
19q13.42	CN431418	Interleukin-11 splice variant
20p11.1	BC036544	mRNA
20p12.1	CB961129	Spliced EST
21q22.11	OLIG2	Oligodendrocyte transcription factor 2
Xp22.33	SHOX	Short stature homeobox

Table 1. **Promoter-associated CpG islands cloned by MCA/RDA from a PV patient.**

Screening analyses by methylated CpG island amplification followed by hybridization to promoter microarrays are under way to identify more methylated CpG islands in erythroleukemic cell lines HEL (carrying JAK2 mutation) and TF-1. Next we will perform MCA/microarray analysis of PV samples as planned in Task 1e.

PROGRESS ON TASK 2

Validation of MCA/RDA results.

Progesterone receptor. We determined methylation levels of in the CpG island at the start of progesterone receptor isoforms A and B (PGR-A, PGR-B) by quantitative bisulfite pyrosequencing in normal controls, ET, PV, and MF patients (Figure 2). Methylation of PGR-A over a 10% threshold was observed in 0/46 controls, 0/59 ET patients, 15/128 (12%) PV patients, and 14/73 (19%) MF patients. Acute myeloid leukemia (AML) patients showed markedly higher methylation frequency (23/33 patients, 70%). Threshold of 15% was used for methylation of PGR-B. Methylation above this threshold was seen in none of 45 controls, 3/59 (5%) ET patients, 18/132 (14%) PV patients, 13/73 (18%) MF patients, and in 22/36 (61%) AML patients. Methylation levels of PGR-A and PGR-B in AML were significicantly higher than in controls ($P < 0.001$; Dunn's multiple comparison nonparametric test).

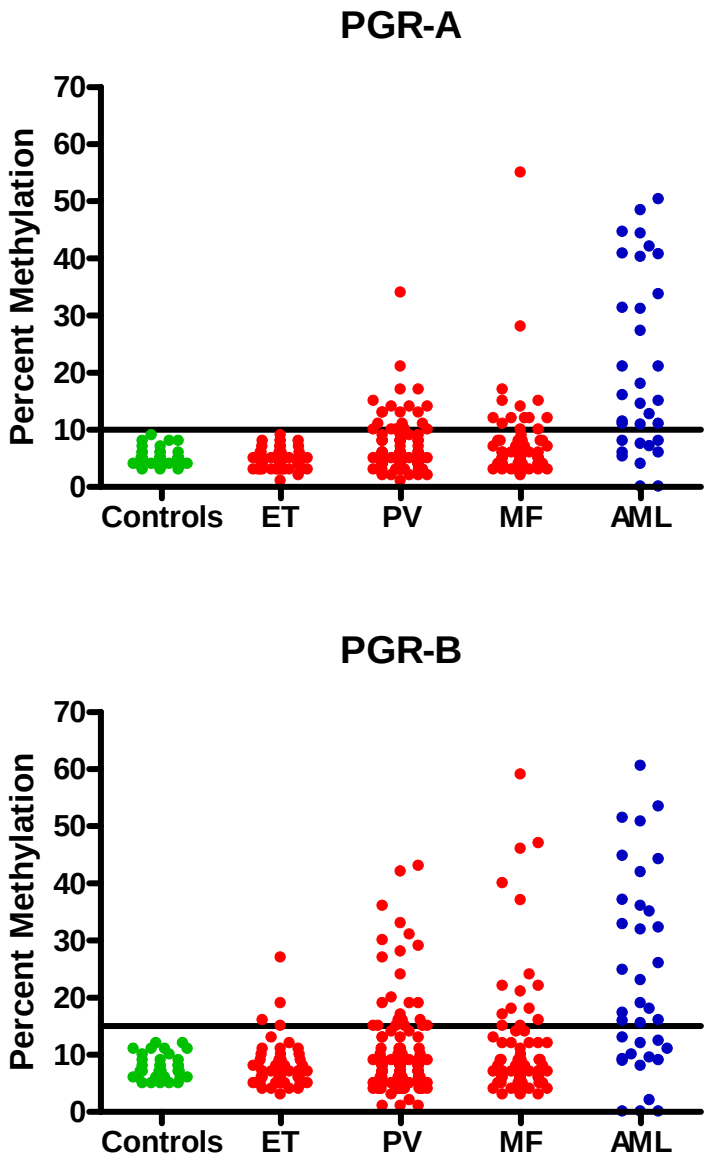

Figure 2. Quantitative determination of methylation levels in CpG islands of progesterone receptor isoforms A (PGR-A) and B (PGR-B).

CDH13. Heart cadherin precursor (CDH13) was another CpG island recovered by MCA/RDA in PV. Methylation of CDH13 over a 10% threshold was observed in 1/46

(2%) controls, 4/59 (7%) ET patients, 19/130 (15%) PV patients, 19/72 (26%) MF patients, and in 12/36 (33%) AML patients.

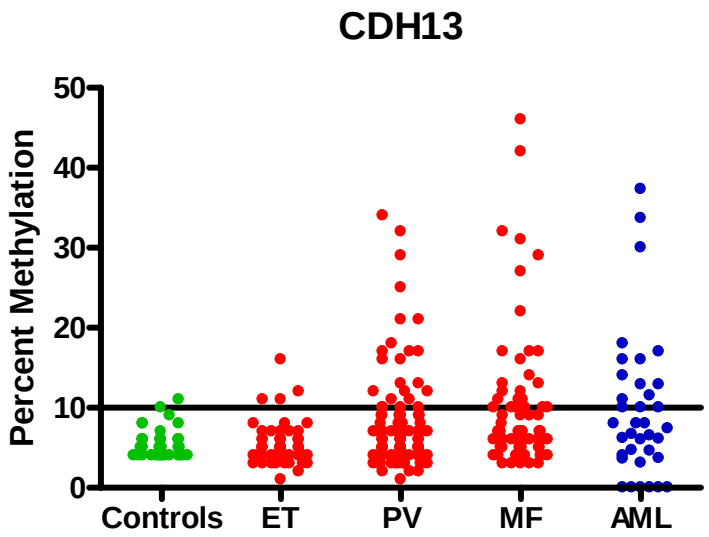

Figure 3. Quantitative determination of methylation levels in CpG island at the start of heart cadherin precursor gene (CDH13).

Methylation levels for PGR-A, PGR-B and CDH13 were normalized by a Z transformation and methylation Z-scores were calculated by the following formula:

Z = (methylation value – mean methylation / standard deviation)

Average Z-scores were calculated for each sample. Methylation Z-score values were significantly increased in MF ($P < 0.01$) and AML patients ($P < 0.001$) when compared to controls (Figure 4).

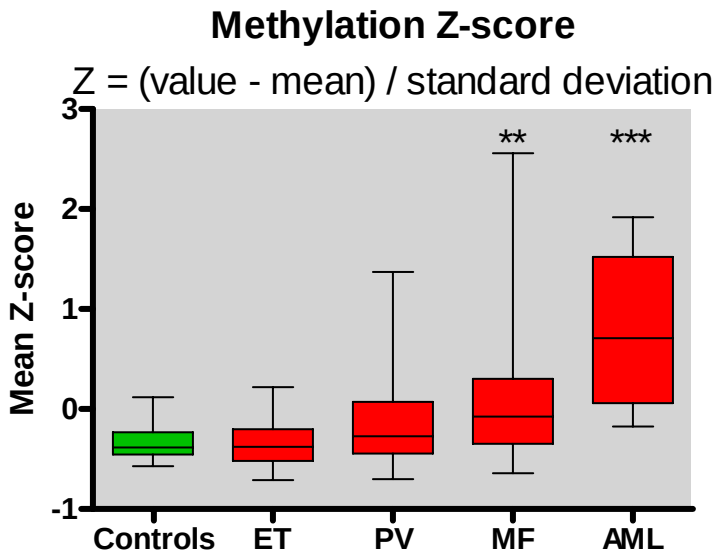

Figure 4. Methylation Z-scores are increased in MF and AML patients.

Other genes. Thirteen additional CpG islands recovered by the initial MCA/RDA screen in PV were validated by bisulfite pyrosequencing or combined bisulfite restriction analysis (COBRA). The results are summarized in Table 2. Four genes (CNR1, OLIG2, SNCB and TERT) and one mRNA (BC043593) showed methylation in leukemic cell lines while methylation in normal controls was not increased. We will screen larger numbers of PV patients to determine methylation frequencies for these genes. Two genes, CHST11 and GALNACT2 were not methylated in controls, PV patients or leukemic cell lines. BC036544 mRNA and JMJD1B gene were methylated in all tested groups. LNK gene was universally methylated at the CpG island at exon 2. This region was detected by MCA/RDA. Pyrosequencing assay designed for CpG island at exon 1 revealed the absence of methylation in all tested samples.

Frequency of methylation over 10%			
Gene	**Controls**	**PV patients**	**Leukemic lines**
BC036544	2/2	7/7	13/13
BC043593	0/10	0/7	4/5
CAPON	0/9	0/7	1/5
CHST11	0/2	0/7	0/12
CNR1	0/3	0/4	5/6
DGKQ	0/2	0/6	2/13
GALNACT2	0/2	0/5	0/6
JMJD1B	2/2	4/7	9/13
LNK exon 1	0/10	0/7	0/10
LNK exon 2	10/10	7/7	10/10
OLIG2	0/2	0/4	6/6
SNCB	0/8	0/7	4/6
TERT	0/10	1/7	5/5

Table 2. Methylation frequencies of CpG islands recovered by MCA/RDA determined by bisulfite pyrosequencing or COBRA analysis.

Progress on Task 3

To assess the functional significance of progesterone receptor silencing, we explored the effect of mifepristone, a PGR antagonist, on *in vitro* response of BFU-E erythroid progenitors to erythropoietin. Mifepristone at 10^{-6} M concentration increased the sensitivity of BFU-E progenitors from normal blood to low concentrations of erythropoietin (60-250 mU/ml) suggesting that disabling of PGR may increase the response of hematopoietic cells to proliferative stimuli (Figure 5).

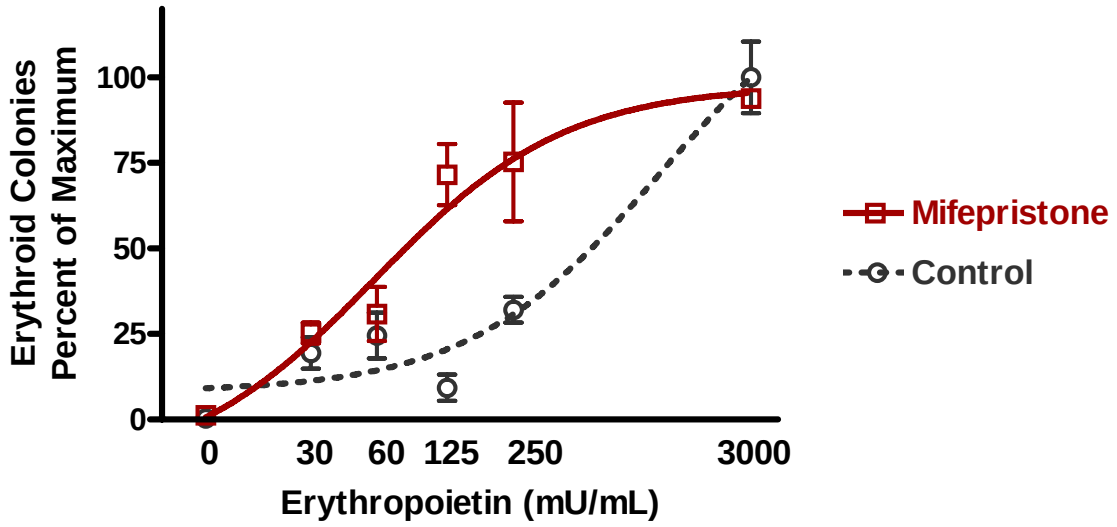

Figure 5. Progesterone receptor antagonist mifepristone increased the sensitivity of erythroid progenitors BFU-E to erythropoietin. Results of four independent experiments were combined. Error bars show standard error of the mean.

KEY RESEARCH ACCOMPLISHMENTS

- Cloned 19 unique CpG islands in promoter/exon-1 regions of 15 known genes, and 4 predicted genes and annotated mRNAs as potentially hypermethylated in PV.
- Confirmed increased methylation of progesterone receptor and cadherin precursor in a subset of PV, MF and AML patients.
- Showed that a functional block of progesterone receptor in normal erythroid cells increases their sensitivity to proliferative stimulation by erythropoietin.

REPORTABLE OUTCOMES

Meeting presentations

Poster, Methylation of progesterone receptor promoter-associated CpG island in polycythemia vera and related myeloproliferative disorders. American Society of Hematology 47th Annual Meeting, Atlanta, GA, December 12, 2005

Poster, DNA methylation as an epigenetic factor in the development and progression of polycythemia vera. PRMRP Military Health Research Forum, San Juan, PR, May 1-4, 2006

Meeting abstracts published

Jelinek J, Verstovsek S, Bueso-Ramos CE, Prchal JT, Issa JPJ. Methylation of progesterone receptor promoter-associated CpG island in polycythemia vera and related myeloproliferative disorders. Blood 2005;106:979a-980a (Appendix 1)

Prchal JT, Chang KT, Jelinek J, Guan Y, Gaikwad A, Issa JP, Liu E. In vitro expansion of polycythemia vera progenitors favors expansion of erythroid precursors without JAK2 V617F mutation. Blood 2005;106:979a (Appendix 2)

Manuscript published

Oki Y, Jelinek J, Beran M, Verstovsek S, Kantarjian HM, and Issa JPJ. Mutation and promoter methylation status of NPM1 in myeloproliferative disorders. Haematologica, 2006;91:1147-1148 (Appendix 3)

Manuscript submitted

Nussenzveig RH, Swierczek S, Jelinek J, Gaikwad A, Liu E, Verstovsek S, Prchal JF, and Prchal JT. A Novel and Quantitative Real-Time AS-PCR Indicates that JAK2V617F is not the PV Initiating Mutation.

Manuscript in preparation

Jelinek J, He R, Bueso-Ramos CE, Verstovsek S, Prchal JT, Issa JPJ. Methylation of progesterone receptor CpG islands in polycythemia vera, myelofibrosis and leukemia.

CONCLUSIONS

Our data show that *PGR* and *CDH13* CpG islands are methylated in a significant proportion of PV and MF patients and in leukemia. Silencing of these genes by methylation may contribute to disease development by altering the response of hematopoietic cells to proliferative stimuli or their interactions with stroma.

"SO WHAT:"

Epigenetic silencing by cytosine methylation in selective CpG islands may play a role in the development of myeloproliferative disorders. The hypomethylating drug decitabine may be considered for clinical trials in patients non-responding to conventional treatment.

REFERENCES

1. Kutti J, Ridell B. Epidemiology of the myeloproliferative disorders: essential thrombocythaemia, polycythaemia vera and idiopathic myelofibrosis. Pathol Biol (Paris). 2001;49:164-166.
2. Spivak JL. Diagnosis of the myeloproliferative disorders: resolving phenotypic mimicry. Semin Hematol. 2003;40:1-5.
3. Passamonti F, Rumi E, Pungolino E, et al. Life expectancy and prognostic factors for survival in patients with polycythemia vera and essential thrombocythemia. Am J Med. 2004;117:755-761.
4. Finazzi G, Caruso V, Marchioli R, et al. Acute leukemia in polycythemia vera. An analysis of 1,638 patients enrolled in a prospective observational study. Blood. 2005;105:2264-2270.
5. Marchioli R, Finazzi G, Landolfi R, et al. Vascular and Neoplastic Risk in a Large Cohort of Patients With Polycythemia Vera. J Clin Oncol. 2005;23:2224-2232.
6. James C, Ugo V, Le Couedic JP, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature. 2005;434:1144-1148.
7. Baxter EJ, Scott LM, Campbell PJ, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet. 2005;365:1054-1061.
8. Kralovics R, Passamonti F, Buser AS, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med. 2005;352:1779-1790.
9. Levine RL, Wadleigh M, Cools J, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell. 2005;7:387-397.
10. Zhao R, Xing S, Li Z, et al. Identification of an acquired JAK2 mutation in polycythemia vera. J Biol Chem. 2005;280:22788-22792.
11. Steensma DP, Dewald GW, Lasho TL, et al. The JAK2 V617F activating tyrosine kinase mutation is an infrequent event in both "atypical" myeloproliferative disorders and myelodysplastic syndromes. Blood. 2005;106:1207-1209.
12. Jelinek J, Oki Y, Gharibyan V, et al. JAK2 mutation 1849G>T is rare in acute leukemias but can be found in CMML, Philadelphia chromosome-negative CML, and megakaryocytic leukemia. Blood. 2005;106:3370-3373.
13. Levine RL, Loriaux M, Huntly BJ, et al. The JAK2V617F activating mutation occurs in chronic myelomonocytic leukemia and acute myeloid leukemia, but not in acute lymphoblastic leukemia or chronic lymphocytic leukemia. Blood. 2005;106:3377-3379.
14. Issa JP. Aging, DNA methylation and cancer. Crit Rev Oncol Hematol. 1999;32:31-43.
15. Toyota M, Ho C, Ahuja N, et al. Identification of differentially methylated sequences in colorectal cancer by methylated CpG island amplification. Cancer Res. 1999;59:2307-2312.

APPENDIX 1

Blood (ASH Annual Meeting Abstracts) 2005 106: Abstract 3507

Poster Sessions

Methylation of Progesterone Receptor Promoter-Associated CpG Island in Polycythemia Vera and Related Myeloproliferative Disorders.

Jaroslav Jelinek, MD, PhD[1], Srdan Verstovsek, MD[1], Carlos E. Bueso-Ramos, M.D, PhD[2], Josef T. Prchal, MD[3] and Jean-Pierre J. Issa, MD[1]

[1] Department of Leukemia, University of Texas M.D. Anderson Cancer Center, Houston, TX, USA; [2] Department of Hematopathology, University of Texas M.D. Anderson Cancer Center, Houston, TX, USA and [3] Hematology/Oncology Division, Baylor College of Medicine, Houston, TX, USA.

Abstract

Polycythemia vera (PV), essential thrombocythemia (ET) and idiopathic myelofibrosis (MF) are clonal myeloproliferative disorders (MPD). A recently discovered activating mutation of JAK2 tyrosine kinase has been found in most patients with polycythemia vera (PV), in about half of those with essential thrombocythemia (ET) and myelofibrosis (MF), and in 10–20% patients with chronic myelomonocytic leukemia, Philadelphia-negative CML, atypical or unclassified MPD and megakaryocytic leukemia. It is not known what other factors determine the disease phenotype of PV, MF, and other MPD, and what factors other than JAK2 lead to disease progression. Very little is known about epigenetic changes in PV. DNA methylation of promoter-associated CpG islands is a well-recognized mechanism of epigenetic silencing used by tumors for evasion from regulatory mechanisms, and it is an alternative to genetic lesions in cancer causation. Using a genome-wide screen for differentially methylated CpG islands, we found methylation of progesterone receptor promoter region (PGR) in PV granulocytes. We then developed pyrosequencing assays for quantitative detection of PGR methylation in bisulfite-treated PCR-amplified DNA. The PGR methylation above normal control levels was observed in ET (2/12 patients, 17%), PV (10/22 patients, 45%), MF (8/12 patients, 67%), and patients with acute myeloid leukemia and antecedent PV (6/7 patients, 86%). We compared the levels of PGR methylation in MPD with the mutation status of JAK2. The *1849G>T JAK2* mutation was present in 16/27 (59%) MPD patients with unmethylated PGR and 21/26 (80%) patients with methylated PGR; the difference not statistically significant; p=0.135. The role of progesterone receptor signaling in hematopoiesis is not known. Using real time quantitative RT-PCR assay for progesterone receptor expression we found detectable levels in granulocytes from 4/5 normal individuals while the expression in granulocytes from 5/5 PV patients was not detectable.

To assess the functional significance of progesterone receptor silencing, we explored the effect of mifepristone, a progesterone receptor antagonist, on the response of BFU-E progenitors to erythropoietin. Mifepristone increased the sensitivity of BFU-E progenitors from normal blood to low concentrations of erythropoietin (60–250 mU/ml) suggesting that disabling of progesterone receptor may increase the response of hematopoietic cells to proliferative stimuli. In conclusion, our data show that PGR methylation is present in half of PV patients and it is even more frequent in MF and PV transformed to AML. Silencing of progesterone receptor by methylation may be an epigenetic change contributing to MPD phenotype and transformation to leukemia.

APPENDIX 2

Blood (ASH Annual Meeting Abstracts) 2005 106: Abstract 3506

Poster Sessions

In Vitro Expansion of Polycythemia Vera Progenitors Favors Expansion of Erythroid Precursors without JAK2 V617F Mutation.

Josef T. Prchal, MD[1,2], Ko-Tung Chang, PhD[1,2,*], Jaroslav Jelinek, MD[3], Yongli Guan[1,*], Amos Gaikwad, PhD[1,*], Jean-Pierre Issa, MD[3] and Enli Liu, MD[1,*]

[1] Medicine-Hematology/Oncology, Baylor College of Medicine, Houston, TX, USA; [2] Dpt of Pathophysiology, School of Medicine, Charles University, Prague, Czech Republic and [3] Department of Leukemia, University of Texas M. D. Anderson Cancer Center, Houston, TX, USA.

Abstract

A single acquired point mutation of JAK2 1849G>T (V617F), a tyrosine kinase with a key role in signal transduction from growth factor receptors, is found in 70%–97% of patients with polycythemia vera (PV). In the studies of tyrosine kinase inhibitors on JAK2 1849G>T (see Gaikwad et all abstract at this meeting) we decided to study the possible therapeutic effect of these agents using native in vitro expanded cells from peripheral blood. To our surprise, the in vitro expansion of PV progenitors preferentially augmented cells without JAK2 1849G>T mutation.

We used a 3 step procedure to amplify erythroid precursors in different stages of differentiation from the peripheral blood of 5 PV patients previously found to be homozygous or heterozygous for the JAK2 1849G>T mutation. In the first step (days 1–7), 106/ml MNCs were cultured in the presence of Flt-3 (50 ng/ml), Tpo (100 ng/ml), and SCF (100 ng/ml). In the second step (days 8–14), the cells obtained on day 7 were re-suspended at 106/ml in the same medium with SCF (50 ng/ml), IGF-1 (50 ng/ml), and 3 units/ml Epo. In the third step, the cells collected on day 14 were re-suspended at 106/ml and cultured for two more days in the presence of the same cytokine mixture as in the step 2 but without SCF. The cultures were incubated at 37oC in 5% CO2/95% air atmosphere and the medium renewed every three days to ensure good cell proliferation. The expanded cells were stained with phycoerythrin-conjugated anti-CD235A (glycophorin) and fluorescein isothiocyanate-conjugated anti-human-CD71 (transferrin receptor) monoclonal antibodies and analyzed by flow cytometry. The cells were divided by their differential expression of these antigens into 5 subgroups ranging from primitive erythroid progenitors (BFU-Es and CFU-Es) to polychromatophilic and orthochromatophilic erythroblasts; over 70% of harvested cells were early and late basophilic erythroblasts. The proportion of JAK2 1849G>T mutation in clonal PV

granulocytes (GNC) before in vitro expansion and in expanded erythroid precursors was quantitated by pyrosequencing (**Jelinek**, Blood in press) and is depicted in the Table.

These data indicate that in vitro expansion of PV progenitors favors expansion of erythroid precursors without JAK2 V617F mutation. Since three PV samples were from females with clonal granulocytes, erythrocytes, and platelets, experiments were underway to determine if the in vitro expanded erythroid cells were clonal PV cells without JAK2 V617F mutation, or derived from polyclonal rare circulating normal hematopoietic progenitors.

The Proportion of JAK2 T Allele

Patients	GNC T Allele (%)	Expanded Cells T Allele (%)
PV1 (Female)	81	10
PV2 (Male)	77	28
PV3 (Male)	44	42
PV4 (Female)	78	19
PV5 (Female)	78	28

Myeloproliferative Disorders

Mutations and promoter methylation status of *NPM1* in myeloproliferative disorders

We determined mutations and promoter methylation status of *NPM1* using pyrosequencing in 199 samples of myeloid neoplasia including myeloproliferative disorders (MPD). The mutations were present in 4% of chronic myelomonocytic leukemia, but not in other MPD or myelodysplastic syndromes. Promoter methylation was rare, and was found in only three samples of MPD.

Haematologica 2006; 91:1147-1148
(*http://www.haematologica.org/journal/2006/08/1147.html*)

Mutations in nucleophosmin 1 gene (*NPM1*, localized on 5q32) were found to be frequent events in acute myeloid leukemia (AML).[1-3] NPM1 likely plays a role as a tumor suppressor in myeloid hematopoiesis, and its haploinsufficiency has been suggested.[4,5] *NPM1* mutations have not been observed in myelodysplastic syndrome (MDS), chronic myeloid leukemia (CML), or lymphoid malignancies.[1] Here we describe a simple and sensitive screening method for *NPM1* mutation using a pyrosequencing assay, which has a detection limit of approximately 5% of mutant alleles.[6] We applied this to 14 leukemic cell line samples (OCI/AML3, ALL1, BJAB, CEM, HEL, HL60, JTAG, Jurkat, K562, KG1, KG1a, ML1, Raji, and TF-1) and 199 samples from patients including 39 AML (including 11 with diploid karyotype and four with 5q or chromosome 5 deletion), 15 Ph-positive and 15 Ph-negative CML, 50 MDS (including ten with 5q or chromosome 5 deletion), 50 chronic myelomonocytic leukemia (CMML), 14 polycythemia vera (PV), 7 essential thrombocythemia (ET) and 9 myelofibrosis (MF). Samples from patients were obtained from peripheral blood or bone marrow mononuclear cells, and all patients gave their consent to the donation of samples. Furthermore, promoter methylation, which can cause gene silencing,[7] of the *NPM1* gene was also analyzed.

The pyrosequencing assay for mutation analyses was the same at the one we had previously utilized for analysis of the *JAK2* mutation.[6] First, exon 12 of the *NPM1* gene was amplified by polymerase chain reaction (PCR) using primers NPM1-F: 5'-TTAACTCTCTGGTGGTAGAATG-3' and biotinylated-NPM1-R: 5'-ACATTTATCAAACACG-GTAGG-3'. Then the biotinylated strand was captured on streptavidin sepharose beads and annealed with a sequencing primer NPM1-S: 5'-TTTTCCAGGC-TATTCAAGAT -3'. Pyrosequencing was performed using PSQ HS 96 Gold SNP Reagents and the PSQ HS 96 pyrosequencing machine (Biotage, Uppsala, Sweden). Programmed polymorphic sites were set at nucleotides 959 (A/C), 960 (G/T) and 964 (G/C) to detect all previously reported mutation variants (Figure 1 A). Mutations are

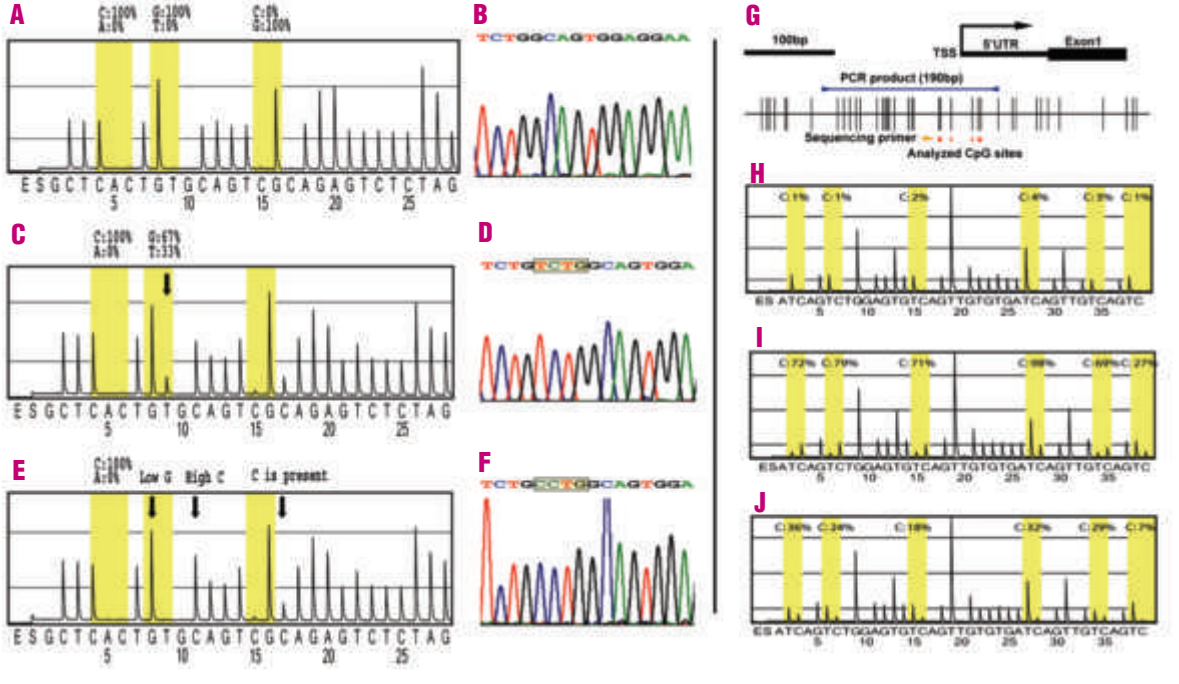

Figure 1. A-F. Mutation assays. Expected wild type sequence in this region is CTCTGGCAGTGGAGGAAGTCTCTTTAAG. **A.** A pyrogram of a normal sample showing only wild type allele. **B.** Cloning and sequencing confirmed a wild type sequence only. **C.** A pyrogram in a patient with CMML, showing an abnormal T peak at position 960, suggesting the presence of a mutant allele. **D.** Cloning and sequencing confirmed the presence of mutation A (960-961insTCTG). **E.** A pyrogram with multiple abnormal peaks in another patient with CMML, suggesting the presence of a mutant allele. F. Cloning and sequencing confirmed the presence of mutation D (960-961insCCTG). **G.** *Schematic map of the promoter region*. CpG sites at -21, -19, -8, 15, 22, and 24 bases from the transcription start site were analyzed. Vertical bars indicate CpG sites. TSS indicates the transcription start site; UTR: untranslated region. **H-J.** *Methylation assays*. The expected sequence in this region is YGYGGGGAGTTTGYGTTTTTTTTTTGGTGTGATTTYGTTTTGYGY (Y=T or C). **H.** A pyrogram of a normal sample showing no methylation. **I.** A pyrogram of SssI methylase treated normal control DNA (methylation positive control), showing an average methylation of 69% (average of six CpG sites). **J.** A pyrogram of a sample from a patient with myelofibrosis, showing an average methylation of 24%.

Table 1. Summary of the mutation and methylation analyses.

Disease	*N*	*Prevalence of NPM1 mutation*	*Prevalence of NPM1 methylation*
Leukemic cell lines	14	1 (OCI/AML3)	2 (CEM, ML1)
Acute myeloid leukemia	39	5 (13% of all cases, 45% of diploid cases)	0 (0%)
Chronic myeloid leukemia	15	0 (0%)	0 (0%)
Myelodysplastic syndrome	50	0 (0%)	0 (0%)
CMML	50	2 (4%)	0 (0%)
Ph-negative CML	15	0 (0%)	0 (0%)
Polycythemia vera	14	0 (0%)	1 (7%)
Essential thrombocytopenia	7	0 (0%)	0 (0%)
Myelofibrosis	9	0 (0%)	2 (22%)

CML: chronic myeloid leukemia; CMML: chronic myelomonocytic leukemia.

detected as abnormal pyrogram patterns (pyrosequencing peaks) compared to the wild type pattern. When a mutation was indicated, PCR was repeated using primers without the biotin tag, then cloned in a plasmid and sequenced at the M.D. Anderson Cancer Center DNA Core Facility using ABI Big Dye terminator cycle sequencing chemistry to confirm the mutation.

First, leukemic cell lines and primary AML samples were analyzed to confirm the validity of the mutation analysis. Among cell line samples, only OCI/AML3 showed the *NPM1* mutation, consistent with a previous report.[12] Among 39 AML samples, five with diploid karyotype showed *NPM1* mutations, all as forms of insertion between positions 959 and 960.[8] The frequency of this mutation in AML patients with diploid karyotype is consistent with that in previous reports.[1-3] We did not observe mutations in other positions. The *NPM1* mutation was not observed in Philadelphia chromosome (Ph) positive-CML and MDS (excluding CMML) samples. Among patients with MPD, *NPM1* mutations were observed in two of 50 cases of CMML (Figure 1C, 1E) but in no cases of Ph-negative CML, ET, PV, or MF (Table 1). The patient with mutation A (960-961insTCTG) was a 78-year old male with CMML, with a white blood count of 15×10^9/L and 13% peripheral monocytes. Bone marrow showed CMML, with 14% blasts and normal karyotype. This patient was treated with decitabine and achieved a complete remission, when *NPM1* mutation was undetectable. Mutation D (960-961insCCTG) was detected in a 77-year old female with CMML. Bone marrow showed 6% blasts and a normal karyotype. The woman had a peripheral white blood cell count of 6.4×10^9/L, with 1% blasts and 17% monocytes. Twelve months later, the patient developed AML with *NPM1* mutation D.

We used the bisulfite pyrosequencing method for methylation analyses.[9] The promoter region of *NPM1* in bisulfite-treated DNA[10] was amplified by PCR using primers NPM1-Bis-F: 5'-AAGGAGTGGGGTTGAAAAG-3' and biotinylated-NPM1-Bis-R: 5'-CCCTACTC-CAAAAAACAACC-3'. After PCR, T/C polymorphisms, corresponding to unmethylated and methylated cytosines in the original DNA, at -21, -19, -8, 15, 22, and 24 bases from the transcription start site, were analyzed with pyrosequencing,[13] using sequencing primer NPM1-Bis-S: 5'-GAGATTTTTAGGGTTTATATATAAG-3' (Figure 1 G). The methylation percentage was calculated by the average of the degree of methylation at six CpG sites formulated in pyrosequencing. In cell lines, CEM and ML1 showed a low degree of methylation (average 29% and 36%, respectively). However, the *NPM1* expression assay (real-time PCR using Hs01576587_g1 [Applied Biosystems] and GAPDH as internal controls) showed no evidence of gene silencing, when compared to other cell lines (*data not shown*). In samples from patients, promoter hypermethylation was only observed in two cases with MF and one with PV (50%, 24% [Figure 1J] and 24%, respectively), who all had a diploid karyotype.

In conclusion, we screened samples of MDS and MPD for mutations of *NPM1*, and detected mutations in 4% of patients with CMML. *NPM1* mutations were not observed in MDS or other MPD. Promoter hypermethylation of *NPM1* is rare in myeloid neoplasms. *NPM1* mutations, methylation and 5q deletion were not found simultaneously, although our study included a limited number of patients. The significance of promoter hypermethylation needs to be investigated further.

Yasuhiro Oki, Jaroslav Jelinek, Miloslav Beran, Srdan Verstovsek, Hagop M. Kantarjian, Jean-Pierre J. Issa

Department of Leukemia, The University of Texas, M.D. Anderson Cancer Center, Houston, TX 77030 USA

Key words: NPM1, myeloproliferative disorder, chronic myelomonocytic leukemia, methylation.

Correspondence: Yasuhiro Oki, M.D., Department of Leukemia, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd, Box 10, Houston, TX 77030, USA. Phone: international +1.713.7922740. Fax: international +1.713.7944297. E-mail: yooki-tky@umin.ac.jp

References

1. Falini B, Mecucci C, Tiacci E, Alcalay M, Rosati R, Pasqualucci L, et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. N Engl J Med 2005;352:254-66.
2. Cazzaniga G, Dell'Oro MG, Mecucci C, Giarin E, Masetti R, Rossi V, et al. Nucleophosmin mutations in childhood acute myelogenous leukemia with normal karyotype. Blood 2005;106:1419-22.
3. Suzuki T, Kiyoi H, Ozeki K, Tomita A, Yamaji S, Suzuki R, et al. Clinical characteristics and prognostic implications of NPM1 mutations in acute myeloid leukemia. Blood 2005;106:2854-61.
4. Berger R, Busson M, Baranger L, Helias C, Lessard M, Dastugue N, et al. Loss of the NPM1 gene in myeloid disorders with chromosome 5 rearrangements. Leukemia 2006;20:319-21.
5. Grisendi S, Bernardi R, Rossi M, Cheng K, Khandker L, Manova K, et al. Role of nucleophosmin in embryonic development and tumorigenesis. Nature 2005;437:147-53.
6. Jelinek J, Oki Y, Gharibyan V, Bueso-Ramos C, Prchal JT, Verstovsek S, et al. JAK2 mutation 1849G>T is rare in acute leukemias but can be found in CMML, Philadelphia chromosome-negative CML, and megakaryocytic leukemia. Blood 2005; 106:3370-3.
7. Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP. Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res 1998;72:141-96.
8. Quentmeier H, Martelli MP, Dirks WG, Bolli N, Liso A, Macleod RA, et al. Cell line OCI/AML3 bears exon-12 NPM gene mutation-A and cytoplasmic expression of nucleophosmin. Leukemia 2005; 19:1760-7.
9. Colella S, Shen L, Baggerly KA, Issa JP, Krahe R. Sensitive and quantitative universal Pyrosequencing methylation analysis of CpG sites. Biotechniques 2003;35:146-50.
10. Clark SJ, Harrison J, Paul CL, Frommer M. High sensitivity mapping of methylated cytosines. Nucleic Acids Res 1994;22:2990-7.